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Relative transcript abundance in porcine cumulus cells collected from different size follicles

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ABSTRACT

Bi-directional communication between the oocyte and surrounding cumulus cells (CCs) is essential for the production of competent oocytes. Previous studies have analyzed the relative transcript abundance in oocytes derived from small follicles (SF: <3 mm diameter) and medium follicles (MF: 3-6 mm diameter) to determinate the potential use of oocytes from SF in assisted reproductive technologies (ART). The aim of this study was to examine the relative transcript abundance in CCs obtained from cumulus-oocyte complexes (COCs) from SF and MF. Nine genes were selected according to its importance for developmental competence: AT-rich interaction domain 1B (ARID1B), bone morphogenic protein receptor 2 (BMPR2), CD44, follicle-stimulating hormone receptor (FSHR), follistatin (FST), inhibin beta-A (INHBA), luteinizing hormone receptor (LHR), nuclear receptor subfamily 2 group F member 6 (NR2F6) and vascular endothelial growth factor A (VEGFA); and its expression was analyzed by RT-qPCR. Results showed significant differences in five genes, the relative transcript abundance of SF-derived CCs was lower for INHBA, whereas it was higher for FSHR, FST, LHR and NR2F6 compared to MF-derived CCs. In conclusion, we present, for the first time, a detailed description of gene activity in the porcine cumulus cells from different size follicles improving our understanding of oocyte biology and will provide new markers that signal viable and competent oocytes.

Keywords: pig, cumulus cells, gene expression, follicle size, ARID1B, BMPR2, CD44, FSHR, FST, INHBA, LHR, NR2F6, VEGFA.

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INTRODUCTION

Cumulus cells (CCs) surround growing oocytes and support their growth and development. It has been well documented that bi-directional communication between the oocyte and the cumulus cells is essential for the production of competent oocytes in several species such as mice (Cross & Brinster, 1970), rats (Dekel & Beers, 1980), bovine (Zhang et al., 1995), porcine (Ferré et al., 2016) and humans (Ebner et al., 2006; Goud et al., 1998). Furthermore, it has been shown in pigs that oocyte competence to achieve the nuclear maturation is not only related with the presence of CCs, but also the timing of oocyte decumulation (Ferré et al., 2016; Ferré et al., 2019).

Cumulus-oocyte complexes (COCs) derived from medium follicles (MF: 3-6 mm in diameter) of prepubertal gilt's ovaries are routinely used for *in vitro* embryo production (IVP) in pigs (Day & Funahashi, 1996; Funahashi & Day, 1997). However, the number of MF on the surface of an ovary is very limited, compared to the number of SF (<3 mm in diameter) (Knox, 2005; Morbeck et al., 1992); and it is well-known that the rate of SF-derived oocytes that reached the metaphase-II (MII) stage after *in vitro* maturation is lower than that of MF-derived oocytes (Bagg, et al., 2007; Ferré et al., 2016; Ferré et al., 2019; Kohata et al., 2013; Marchal et al., 2002; Yoon et al., 2000). Nevertheless, few studies have examined the possibility of using SF-derived oocytes for assisted reproductive technologies (ART) at a large scale.

Our study analyzed the relative transcript abundance in porcine oocytes derived from SF and MF of prepubertal gilt's ovaries to determinate the potential use of SF-derived ones in ART, showing that MII oocytes from SF and MF had similar *in vitro* fertilizability and relative transcript abundance of key genes related with oocyte maturation, fertilization, maternal effect and anti-apoptosis (Kohata et al., 2013).

Therefore, the aim of this study was to examine the relative transcript abundance of CCs collected from SF- and MF-derived COCs to find out whether its abundance was similar. The studied genes are related to key biological events such as hormonal receptors (BMPR2, LHR, FSHR, NR2F6), genes related with oocyte maturation (INHBA and FST), CCs expansion and oocyte meiotic resumption (CD44), gap-junction communication (ARID1B), and an angiogenic factor (VEGFA).

Materials and methods

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The porcine ovaries used in this study were obtained from discarded entrails just after slaughter of gilts for commercial meat production at an abattoir. Therefore, the current study was not included in the category of animal experiments that require approval by the Ethics Committee.

Chemicals and culture media

NaCl, KCl, $MgCl_2•6H_2O$ andKH_2PO_4 were obtained from Nacalai Tesque Inc. (Japan), and $CaCl_2•2H_2O$ was purchased from Ishizu Pharmaceutical Co., Ltd. (Japan). Unless otherwise specified, other chemicals were purchased from Sigma Aldrich (Japan).

The medium used for collecting and washing COCs was modified TL-HEPES-PVA, which comprised 114 mM NaCl, 3.2 mM KCl, 2 mM $NaHCO_3$, 0.34 mM KH_2PO_4, 10 mM Na-lactate, 0.5 mM $MgCl_2•6H_2O$, 2 mM $CaCl_2•2H_2O$, 10 mM HEPES, 0.2 mM Na-pyruvate, 12 mM sorbitol, 0.1% (wt/vol) polyvinyl alcohol (PVA), 25 mg/mL gentamicin, and 65 mg/mL potassium penicillin G.

Collection of cumulus cells, RNA isolation, and cDNA synthesis

Ovaries were collected from slaughtered prepubertal gilts (5-6 months old, crossed-breed) at a local abattoir and transported to the laboratory in 0.9% NaCl containing 75 mg/mL potassium penicillin G and 50 mg/mL streptomycin sulfate within 5 hours. COCs were aspirated from SF (<3 mm) and MF (3-6 mm) on the surfaces of the ovaries using an 18-gauge needle connected to a disposable 10-mL syringe, and washed three times with modified TL-HEPES-PVA medium at room temperature (25 ºC) (Funahashi et al., 1997). Thereafter, the COCs were denuded mechanically by vigorous pipetting through a narrow-bore micropipette, the media containing the CCs (CCs from 30 COCs per replicate and group) was collected and washed twice in PBS-PVA by centrifugation (at 200 x *g* for 10 min). For each experimental group, total RNA from four or five biological replicates (containing CCs from 30 COCs each) was isolated using the PicoPure RNA Isolation Kit (Arcturus Bioscience, USA) according to the manufacturer's instructions. Then, single reverse transcription was performed with the SuperScript First-Strand Synthesis System kit for RT-PCR (Invitrogen-Life Technologies, USA), following the instructions from producers.

Gene expression analysis

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Total DNAse-treated target cDNA was quantified by real-time PCR using a LightCycler 480 SybrGreen I Master in a Lightcycler 480 II system (Roche, Japan) using a cDNA amount of 2 µl and specific primers for each gene (Table 1). Forty-five PCR cycles (95 ºC for 30 seconds, 58 ºC for 30 seconds, and 72 ºC for 20 seconds) were followed by acquisition of the melting curve. For each sample, the median value of PCR triplicates was considered, and data was normalized to the median value for RPL19, a house-keeping gene used as internal control. In addition, to ensure the morphology of germinal vesicle (GV) just after collection of COCs, the oocytes were denuded, fixed for 15 min with 0.5% (v/v) glutaraldehyde in PBS and stained for 30 min with 0.01 mM Hoechst 33342 (Funahashi & Day, 1993). Nuclear stage was classified into GV-0, GV-I, GV-II, GV-III, GV-IV and GV breakdown (GVBD) or diacinesis (Funahashi et al., 1997; Ye et al., 2002).

Statistical analysis

Data are presented as the mean ± SEM. Data were analysed by one-way ANOVA with size of ovarian follicles (small and medium) as fix factor. When ANOVA revealed a significant effect, values were compared by the post hoc Tukey test. A P-value <0.05 was taken to denote statistical significance.

RESULTS

Nuclear Morphology of SF- and MF-derived oocytes

When COCs were collected from SF and MF to analyse the relative transcriptional levels in the CCs, a majority of SF-derived oocytes had the GV-0 nuclear morphology (55.7 ± 5.1%), whereas MF-derived ones were mostly the GV-II morphology (65.3 ± 4.8 %; $P < 0.001$, Table 2). These results showed that the GV morphology in MF-derived oocytes were slightly more advanced as compared with that in SF-derived ones. There were no significant differences between the incidences of SF- and MF-derived oocytes with another GV morphology (GV-1, GV-3 or GV-4).

Relative abundance of mRNA in SF- and MF-derived CCs

It was studied if the transcript abundance of ARID1B, BMPR2, CD44, FSHR, FST, INHBA, LHR, NR2F6 and VEGFA genes in CCs changed during follicular development, with two experimental groups, SF- and MF-derived CCs. Relative transcriptional levels of FSHR, FST, LHR and NR2F6 were significantly higher ($p < 0.05$) in SF-derived CCs

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than MF-derived ones, whereas the level of INHBA was lower ($p < 0.01$) in SF-derived oocytes (Fig. 1). On the other hand, there are not significant differences in the relative transcript abundance of ARID1B (p=0.281), BMPR2 (p=0.116), CD44 (p=0.057) and VEGFA (p=0.429) between SF- and MF-derived oocytes.

DISCUSSION

In this study, the transcript abundance of CCs obtained from SF and MF of prepubertal gilts' ovaries was investigated. Results confirmed that meiotic progression of MF-derived oocytes was closer to reach MII stage than that of SF, what agrees with previous reports (Ferré et al., 2016). In light of this observation, perhaps it would be interesting to consider that COCs derived from SF might need a somewhat longer maturation period than those from MF. COCs derived from MF are commonly used for IVP of pig embryos (Day & Funahashi, 1996; Funahashi & Day, 1997), even though the number of SF is significantly larger (Knox, 2005; Morbeck et al., 1992). Therefore, it would be a big advantage for porcine IVP to use SF-derived COCs, grown and matured *in vitro* (Hirao et al., 1994).

Our results show significant differences in CCs gene expression related to follicle size, in five genes. The relative transcript abundance of INHBA was lower in CCs derived from SF versus MF. Whilst, that of FSHR, FST, LHR and NR2F6 was higher in CCs derived from SF versus MF. When previous studies analysed the transcript abundance of 8 genes in SF- and MF-derived oocytes, a significant effect of follicle diameter was found only in one gene (MOS) (Kohata et al., 2013).

INHBA function is being suggested to be related with oocyte differentiation and follicle growth (Ireland et al., 2009; Kempisty et al., 2015), previous studies have demonstrated a higher expression in porcine MF-derived oocytes, as compared with SF of INHBA gene after IVM (Kempisty et al., 2012) and protein prior and after IVM (Kempisty et al., 2015); and in MII bovine oocytes than GV ones (Leal, et al., 2012). Our results found that INHBA expression was along the same line in CCs.

FSH is essential for follicular growth and prevention of atretic antral follicles (Wang & Greenwald, 1993) and LH regulates meiotic resumption (Jaffe & Egbert, 2017). FSH and LH receptors are expressed on the surface of CCs (Shimada et al., 2003; Vigone et al., 2015). In bovine, previous studies have demonstrated a down regulation of FSHR in CCs derived from MII oocytes compared to GV oocytes (Leal et al., 2011) and in ovine

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FSHR and LHR expression showed a downregulation in CCs derived from matured, compared to immature oocytes (Dhali et al., 2017). In our work, the results in pigs coincide with those obtained in ruminants.

FST is related with oocyte developmental competence (Patel et al., 2007), being its expression higher in bovine MII-oocytes versus GV-oocytes (Leal et al., 2011). However, FST is down-regulated in CCs derived from bovine MII- oocytes compared to GV-oocytes (Leal et al., 2011). Similar results are obtained in our study for porcine CCs.

NR2F6 inhibits LHR (Zhang & Dufau, 2000) and has been detected in human CCs, being up-regulated in patients with clinical pregnancy (Iager et al., 2013); however, as far as we know there are no studies related to its expression in SF- and MF-derived CCs. We found a higher expression in SF-derived CCs in the current study.

In summary, our findings support the available literature and provide for the first time detailed information on the transcriptomic activity of the CCs surrounding COCs from different follicle size. This study lays the ground for future functional studies that can enhance our understanding of porcine oocyte maturation and progress in the use of SF-derived COCs to be used in porcine IVP.

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Conflict of Interest Statement: The authors declare no conflicts of interest.

Data availability: The data that supports the results of this study are available upon reasonable request.

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Table 1. Sequences of the primers used for quantitative real-time PCR.

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Gene	Forward 5´-3´ and Reverse 5´-3´	Product size (bp)	GenBank accesion number
ARID1B	CTACGTTCAATCTCTCCCAG	158	XM_021071872.1
	GCTGTCATCTCTCTTTACCG		
BMPR2	ACATGACAACATTGCTCGCT	113	NM_001204900.1
	AATACTTGCATAGAGATCCATT		
CD44	GAGGATGATATGAGCAGTGG	191	Designed from: Kimura *et al*., 2002 10.1095/biolreprod66.3.707
	GGTGCGTAGTAGTCGGAAG		
FSHR	CCACTGCTGTGCCTTTGC	161	NM_214386.3
	CAAATTCTTTGGCTAAACTGG		
FST	CCTATGAGGGAAAGTGTATC	153	NM_001003662.1
	CACAGACAGGCTCCTCAG		
INHBA	GATCATCACCTTCGCGGAA	118	NM_214028.1
	GACTTTCAGGAAGAGCCAG		
LHR	GAAAGCACAGCAAGGAGAC	121	JN120794.1
	GAGCACATTGGAGTGTCTTG		
NR2F6	GGAGCAGGTGGACAAGCTA	99	XM_021083574.1
	GAGAGTCCACAGGCATCAG		
RPL19	TGCTCGAATGCCTGAGAAG	116	XM_003131509.5
	GGTACAGACTGTGATACATG		
VEGFA	GTCTGGAGTGTGTGCCCA	104	XM_013977975.1
	GTGCTGTAGGAAGCTCATC		

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Table 2. Nuclear status of pig oocytes collected from small (SF) and middle (MF) ovarian follicles from which cumulus cells were obtained for gene analysis. Data from 4 replicates and expressed as mean±SEM.

		Nuclear stage					
Group	N	GV-0 (%)	GV-I (%)	GV-II (%)	GV-III (%)	GV-IV (%)	GVBD (%)
SF	**97**	55.7 ± 5.1 a	18.6 ± 4.0	12.4 ± 3.4 a	11.3 ± 3.2	1.0 ± 1.0	2.1 ± 1.5

MF	**98**	4.1 ± 2.0 b	20.4 ± 4.1	65.3 ± 4.8 b	21.4 ± 4.2	5.1 ± 2.2	0
P value		<0.001	0.746	<0.001	0.058	0.101	0.155

Different letters in the same column indicate differences between groups.

Figure legends:

Figure 1. Gene expression in cumulus cells collected from small (SF) and medium (MF) ovarian follicles. The ratio to the mean value in cumulus cells obtained from SF-derived COCs is represented. Data from 4-5 replicates and expressed as mean ± SEM. Different letters indicate significant differences between groups (P<0.05).

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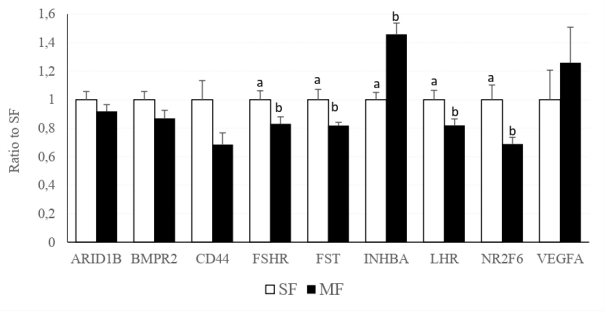

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